Please cite this article as: Joo and Hak, (2018). *In vitro* Nitrate Reductase Activity Assay from *Arabidopsis* Crude Extracts, *Bio-protocol* 8 (7): e2785. DOI: 10.21769/BioProtoc.2785.

bio-protocol www.bio-protocol.org/e2785 Vol 8, Iss 07, Apr 05, 2018
DOI:10.21769/BioProtoc.2785

In vitro Nitrate Reductase Activity Assay from *Arabidopsis* Crude Extracts

Joo Yong Kim[1] and Hak Soo Seo[1, 2, *]

[1]Department of Plant Science and Research Institute of Agriculture and Life Sciences, Seoul National University, Seoul, Korea; [2]Plant Genomics and Breeding Institute, Seoul National University, Seoul, Korea

*For correspondence: seohs@snu.ac.kr

[Abstract] Nitrate reductase (NR) reduces the major plant nitrogen source, NO_3^-, into NO_2^-. NR activity can be measured by its final product, nitrite through its absorbance under optimized condition. Here, we present a detailed protocol for measuring relative enzyme activity of NR from *Arabidopsis* crude extracts. This protocol offers simple procedure and data analysis to compare NR activity of multiple samples.
Keywords: Nitrate reductase, *in vitro* NR activity assay, Nitrite concentration

[Background] Nitrogen is crucial macronutrient required by plants and is mainly absorbed in the form of nitrate. Nitrate reductase is the first enzyme of the nitrogen assimilation in higher plants. Homodimers of plant nitrate reductase catalyze the NAD(P)H-dependent reduction of nitrate to nitrite as follows:

$$NO_3^- + NADH + H^+ \rightarrow NO_2^- + NAD^+ + H_2O$$

Methods to measure NR activity may be a powerful tool to investigate biological factors influencing NR activity (Park *et al*., 2011). Nitrogen assimilation affects the contents of amino acid in plant, thus regulating NR activity could be used for increasing quality of some crop (Croy and Hageman, 1970; Dalling and Loyn, 1977; Ruan *et al*., 1998).

In this protocol, increased nitrite concentrations during limited time in optimized buffer condition are acquired as comparable values. Nitrite concentration is measured by its absorbance at 540 nm through Griess assay. Briefly, nitrite forms a diazonium salt with sulfanilic acid, then N-(1-naphthyl) ethylenediamine dihydrochloride is formed colored azo compound. It is possible to compare the values to determine how samples have different NR activity. Furthermore, the values could be converted to exact increased nitrite concentration through a simple process.

Materials and Reagents

1. 3M™ Micropore™ surgical tape (3M, Micropore™, catalog number: 1530-1)
2. Reaction tube, 1.5 ml (Greiner Bio One International, catalog number: 616201)
3. 150 x 25 mm (d x h) plastic Petri dish (SPL Life Sciences, catalog number: 10151)
4. Cuvette (Ratiolab, catalog number: 2712120)
5. *Arabidopsis* seeds

Please cite this article as: Joo and Hak, (2018). *In vitro* Nitrate Reductase Activity Assay from *Arabidopsis* Crude Extracts, *Bio-protocol* 8 (7): e2785. DOI: 10.21769/BioProtoc.2785.

Vol 8, Iss 07, Apr 05, 2018
DOI:10.21769/BioProtoc.2785

6. Ethanol (Merck, EMSURE®, catalog number: 1009831011)
7. Liquid nitrogen
8. Potassium nitrite (Sigma-Aldrich, catalog number: P8394)
9. Murashige & Skoog medium including vitamins (Duchefa Biochemie, catalog number: M0222)
10. MES monohydrate (Duchefa Biochemie, catalog number: M1503)
11. Potassium hydroxide (Merck, catalog number: 814353)
12. Sucrose (Duchefa Biochemie, catalog number: S0809)
13. Plant agar (Duchefa Biochemie, catalog number: P1001)
14. Tris-HCl (Duchefa Biochemie, catalog number: T1513)
15. Ethylenediaminetetraacetic acid (EDTA) (Sigma-Aldrich, catalog number: EDS)
16. Sodium molybdate dihydrate ($Na_2MoO_4 \cdot 2H_2O$) (Sigma-Aldrich, catalog number: M1003)
17. Flavin adenine dinucleotide disodium salt hydrate (FAD-Na_2) (Sigma-Aldrich, catalog number: F8384)
18. Dithiothreitol (DTT) (Duchefa Biochemie, catalog number: D1309)
19. Bovine serum albumin (BSA) (Merck, Probumin®, catalog number: 821006)
20. 2-Mercaptoethanol (Sigma-Aldrich, catalog number: M6250)
21. Phenylmethylsulfonyl fluoride (PMSF) (Sigma-Aldrich, catalog number: 78830)
22. Sodium nitrate ($NaNO_3$) (Sigma-Aldrich, catalog number: S5506)
23. Sodium phosphate dibasic (Na_2HPO_4) (Bio Basic, catalog number: S0404)
24. Sodium phosphate monobasic, anhydrous (NaH_2PO_4) (Bio Basic, catalog number: SB0878)
25. Nicotinamide adenine dinucleotide (NADH) (Sigma-Aldrich, catalog number: 43420)
26. Hydrochloric acid (HCl) (DAEJUNG CHEMICAL & METALS, catalog number: 4090-4405)
27. Sulfanilamide (Sigma-Aldrich, catalog number: S9251)
28. N-(1-naphthyl) ethylenediamine dihydrochloride (Sigma-Aldrich, catalog number: 222488)
29. MS agar media (see Recipes)
30. Extraction buffer (see Recipes)
31. Reaction buffer (see Recipes)
32. 1% sulfanilamide solution (see Recipes)
33. 0.05% N-(1-naptyl) ethylenediamine hydrochloride (see Recipes)

Equipment

1. Pipette kit (Gilson, model: PIPETMAN® Classic, catalog number: F167300)
2. 2 L flask (DWK Life Sciences, Duran®, catalog number: 21 216 63)
3. *Arabidopsis* growth chamber (Hanbaek, model: HB-301L-3)
4. Stainless steel tweezers
5. Mortar & pestle (Silico & Chemico Porcelain Works, catalog number: J-753)
6. Centrifuge (Thermo Fisher scientific, model: Sorvall™ Legend™ 17, catalog number: 75002431)
7. Spectrophotometer (Biochrom, model: Libra S22)

Please cite this article as: Joo and Hak, (2018). *In vitro* Nitrate Reductase Activity Assay from *Arabidopsis* Crude Extracts, *Bio-protocol* 8 (7): e2785. DOI: 10.21769/BioProtoc.2785.

Vol 8, Iss 07, Apr 05, 2018
DOI:10.21769/BioProtoc.2785

8. Magnetic stirrer (Vision Scientific, model: VS-130SH)
9. Stirring bar
10. pH meter (Fisher Scientific, model: accumet^{TM} AB15)
11. Autoclave (Hanbaek, model: HB-506-6)

Procedure

A. Plant preparation
1. Sterilize *Arabidopsis* seeds with 70% EtOH three times.
2. Plate the seeds on Murashige and Skoog (MS) medium (see Recipes) containing 0.75% agar.
3. Wrap 3M micropore tape around the circumference of each MS plate and store the plates in the dark at 4 °C for 2 days.
4. After 2 days, move the plates to fully automated growth chambers under long day conditions (16 h, 22 °C under light/8 h, 20 °C under dark).

B. Tissue extraction
1. After 15 days, take tissue of interest (0.5 g fresh weight) using stainless steel tweezer.
2. Freeze the samples in liquid nitrogen.
3. Grind samples in liquid nitrogen with chilled mortar and pestle.
4. Add 750 µl of chilled extraction buffer (see Recipes) to ground sample to homogenize.
5. Collect the homogenate into new 1.5 ml tubes through a pipette.
6. Centrifuge at 17,000 *x g* for 5 min.
7. Collect supernatant into new 1.5 ml tubes and discard pellet.

C. Nitrate reductase enzymatic assay
1. Add 150 µl of supernatant to 850 µl of reaction buffer (see Recipes) in a 1.5 ml tube.
   Note: The reaction buffer contains substrate (nitrate) and NADH to initiate reaction.
2. Incubate at room temperature for 2 h.
   Note: To make blank, skip incubation procedure and do next step.
3. Add 200 µl of 1% sulfanilamide and 200 µl of 0.05% N-(1-naphthyl) ethylenediamine hydrochloride by pipetting to stop the reaction.
4. Incubate at room temperature for 15 min.

D. Measure absorbance
1. Transfer 1 ml of the reaction mixture to a cuvette.
2. Measure the absorbance of the reaction mixture at 540 nm.

Please cite this article as: Joo and Hak, (2018). *In vitro* Nitrate Reductase Activity Assay from *Arabidopsis* Crude Extracts, *Bio-protocol* 8 (7): e2785. DOI: 10.21769/BioProtoc.2785.

Vol 8, Iss 07, Apr 05, 2018
DOI:10.21769/BioProtoc.2785

E. Preparation of nitrite standard curve
1. Prepare a nitrite dilution series using potassium nitrite (0, 5, 10, 20, and 40 μM) in 1,250 μl of 0.6x diluted extraction buffer (add 500 μl of ddH_2O to 750 μl of extraction buffer).
   Note: The dilution ratio of extracted buffer for standards is determined based on the assumption that 1 mg fresh weight has a volume of about 1 μl.
2. Add 150 μl of dilution series to 850 μl of reaction buffer (see Recipes) in a 1.5 ml tube.
3. Add 200 μl of 1% sulfanilamide and 200 μl of 0.05% N-(1-naptyl) ethylenediamine hydrochloride by pipetting.
4. Incubate at room temperature for 15 min.
5. Transfer 1 ml of the reaction mixture to a cuvette.
6. Measure the absorbance of the reaction mixture at 540 nm.
7. Generate standard curve for known concentrations of nitrite.

F. Waste disposal
1. All waste must be disposed of in accordance with federal, state and local environmental control regulations.

Data analysis

Values acquired from this protocol are quantity of nitrite molecules. Thus, calculate the NR activity as follows:

$\Delta A = A_{sample} - A_{blank}$

Each sample is assayed in triplicate and ΔA value is blank subtracted from the average of triplicates. ΔA value means the increased concentration of nitrite which is produced during 2 h incubation time (see Step C2) and it could be used as a comparable scale of NR activity. For someone who wants to acquire the exact increased nitrite concentration, calculate the concentration change using the standard curve. The ratio of increasing nitrite content of the sample (nmol NO_2^- g^{-1} FW h^{-1}) using the formula as follows:

[change of nitrite concentration (μM)] x [extracted volume (ml)]/[fresh weight (g)]/[reaction time (h)]

Please cite this article as: Joo and Hak, (2018). *In vitro* Nitrate Reductase Activity Assay from *Arabidopsis* Crude Extracts, *Bio-protocol* 8 (7): e2785. DOI: 10.21769/BioProtoc.2785.

Vol 8, Iss 07, Apr 05, 2018
DOI:10.21769/BioProtoc.2785

Recipes

1. MS agar media
   4.4 g Murashige Skoog basal salt mixture and 0.5 g MES monohydrate in 1 L of deionized water
   Adjust pH to 5.8 with 5 M KOH
   Add 10 g sucrose
   Add 7.5 g plant agar
   Autoclave at 121 °C for 15 min
   Store the medium at room temperature
   Pour media to 150 x 25 mm (d x h) plastic Petri dish
   Poured plated can be stored at 4 °C
2. Extraction buffer
   250 mM Tris-HCl (pH 8.0)
   1 mM EDTA
   1 μM Na_2MoO_4
   5 μM flavin adenine dinucleotide(FAD)
   3 mM dithiothreitol
   1% BSA
   12 mM 2-mercaptoethanol
   250 μM PMSF
   Note: Extraction buffer should be prepared fresh, used immediately and stored on ice.
3. Reaction buffer
   40 mM $NaNO_3$
   80 mM Na_2HPO_4
   20 mM NaH_2PO_4 (pH 7.5)
   0.2 mM NADH
   Note: Reaction buffer without NADH can be stored at 4 °C for several months. Since NADH is added, the buffer should be used immediately.
4. 1% sulfanilamide solution
   Dissolved in 3 M HCl
   Note: This solution is stable for several months.
5. 0.05% N-(1-naphthyl) ethylenediamine hydrochloride
   Dissolved in distilled water. Store the solution in a dark bottle
   Note: This solution is stable for 1 month.

bio-protocol www.bio-protocol.org/e2785 Vol 8, Iss 07, Apr 05, 2018 DOI:10.21769/BioProtoc.2785

Acknowledgments

This work was supported by a grant from the Next-Generation BioGreen 21 Program (Plant Molecular Breeding Center No. PJ01327601), Rural Development Administration, Republic of Korea. This protocol was adapted and modified as described previously (Samuelson and Larsson, 1993; Yu *et al*., 1998, Hachiya and Okamoto, 2016). The authors declare that they have no conflict of interest.

References

1. Croy, L. I. and Hageman, R. H. (1970). Relationship of nitrate reductase activity to grain protein production in wheat. *Crop Science* 10(3): 280-285.
2. Dalling, M. and Loyn, R. (1977). Level of activity of nitrate reductase at the seedling stage as a predictor of grain nitrogen yield in wheat (*Triticum aestivum* L.). *Aus J Agr Res* 28(1):1-4.
3. Hachiya, T. and Okamoto, Y. (2016). Simple spectroscopic determination of nitrate, nitrite, and ammonium in *Arabidopsis thaliana*. *Bio-protocol* 7(10):e2280.
4. Park, B. S., Song, J. T. and Seo, H. S. (2011). *Arabidopsis* nitrate reductase activity is stimulated by the E3 SUMO ligase AtSIZ1. *Nat Commun* 2: 400.
5. Ruan, J., Wu, X., Ye, Y. and Härdter, R. (1998). Effect of potassium, magnesium and sulphur applied in different forms of fertilisers on free amino acid content in leaves of tea (*Camellia sinensis* L). *J Sci Food Agr* 76(3):389-396.
6. Samuelson, M. E. and Larsson, C. M. (1993). Nitrate regulation of zeation riboside levels in barley roots: effects of inhibitors of N assimilation and comparison with ammonium. *Plant Science* 93(1-2):77-84.
7. Yu, X., Sukumaran, S. and Mrton, L. (1998). Differential expression of the *Arabidopsis Nia1* and *Nia2* genes. cytokinin-induced nitrate reductase activity is correlated with increased nia1 transcription and mrna levels. *Plant Physiol* 116(3): 1091-1096.

